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Main Search Page

EXHIBIT C

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Citations available: **9**
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Go to ... Help | Logoff

Citation 1

Authors
Morgan D. Holcomb L. Saad I. Gordon M. Maines M.

Title
IMPAIRED SPATIAL NAVIGATION LEARNING IN **TRANSGENIC** MICE OVER-EXPRESSING HEME OXYGENASE-L

Source
Brain Research. 808(1):110-112, 1998 Oct 12.

Abstract
Transgenic mice expressing heme oxygenase-1 (HO-1) using the **neuron-specific enolase** promoter were impaired in learning the Morris water maze compared to non**transgenic** littermates. The memory of the HO-1 mice for the location of the platform was similarly impaired when tested using a probe trial after 7 training blocks, but performance on visible platform trials was similar for both groups of mice. Importantly, both HO-1 and non**transgenic** mice had normal sensorimotor function, and performed the same on a Y-maze alternation task, highlighting the **specific**ity of memory deficit in the spatial navigation task. These results suggest that carbon monoxide, one product of HO-1 activity, interferes in the development of spatial navigation memory, and may play a role in normal memory function. (C) 1998 Published by Elsevier Science B.V. All rights reserved. [References: 10]

Go to ... Help | Logoff

Citation 2

Authors
Chen JS. Kelz MB. Zeng GQ. Sakai N. Steffen C. Shockett PE. Picciotto MR. Duman RS. Nestler EJ.

Title
TRANSGENIC ANIMALS WITH INDUCIBLE, TARGETED GENE EXPRESSION IN BRAIN

Source
Molecular Pharmacology. 54(3):495-503, 1998 Sep.

Abstract
Several inducible gene expression systems have been developed in vitro in recent years to overcome limitations with traditional **transgenic** mice. One of these, the tetracycline-regulated system, has been used successfully in vivo. Nevertheless, concerns remain about the ability of this system to direct high levels of transgene expression in vivo and to enable such expression to be turned on and off effectively. We report here the generation, using a modified tetracycline-regulated system under the control of the **neuron-specific enolase** promoter, of several lines of mice that direct transgene expression to **specific** brain regions, including the striatum, cerebellum CA1 region of the hippocampus, or deep layers of cerebral neocortex. Transgene expression in these mice can be turned off completely with low doses of doxycycline (a tetracycline derivative) and driven to very high levels in the absence of doxycycline. We demonstrate this tissue-**specific**, inducible expression for three transgenes: those that encode luciferase (a reporter protein) or Delta FosB or the cAMP-response element binding protein (CREB) (two transcription factors). The various lines of **transgenic** mice demonstrate an inducible system that generates high levels of transgene expression in **specific** brain regions and represent novel and powerful tools with which to study the functioning of these (or potentially any other) genes in the brain. [References: 37]

Go to ... Help | Logoff

Citation 3

Authors
Offen D. Beart PM. Cheung NS. Pascoe CJ. Hochman A. Gorodin S. Melamed E. Bernard R. Bernard O.

Title
TRANSGENIC MICE EXPRESSING HUMAN BCL-2 IN THEIR NEURONS ARE RESISTANT TO 6-HYDROXYDOPAMINE AND 1-METHYL-4-PHENYL-1,2,3,6-TETRAHYDROPYRIDINE NEUROTOXICITY

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Source

Proceedings of the National Academy of Sciences of the United States of America. 95(10):5789-5794, 1998 May 12.

Abstract

The protooncogene bcl-2 inhibits **neuron**al apoptosis during normal brain development as well as that induced by cytotoxic drugs or growth factor deprivation. We have previously demonstrated that **neuron**s of mice deficient in Bcl-2 are more susceptible to neurotoxins and that the dopamine (DA) level in the striatum after systemic 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine (MPTP) administration was significantly lower than in wild-type mice, In the present study we have used **transgenic** mice overexpressing human Bcl-2 under the control of **neuron-specific enolase** promoter (NSE-hbcl-2) to test the effects of the neurotoxins 6-hydroxy-dopamine (6-OHDA) and MPTP on **neuron**al survival in these mice. Primary cultures of neocortical **neuron**s from normal and **transgenic** mice were exposed to these dopaminergic neurotoxins, Addition of 6-OHDA resulted in cell death of essentially all **neuron**s from normal mice. In contrast, in cultures generated from heterozygous NSE-hbcl-2 **transgenic** mice, only 69% of the cells died while those generated from homozygous **transgenic** mice were highly resistant and exhibited only 34% cell death. A similar effect was observed with **neuron**s treated with MPP+. Moreover, while the striatal dopamine level after MPTP injections was reduced by 32% in the wild type, the concentration remained unchanged in the NSE-hbcl-2 heterozygous mice. In contrast levels of glutathione-related enzymes were unchanged. In conclusion, overexpression of Bcl-2 in the **neuron**s provided protection, in a dose-dependent manner, against neurotoxins known to selectively damage dopaminergic **neuron**s. This study provides ideas for inhibition of **neuron**al cell death in neurodegenerative diseases and for the development of efficient neuroprotective gene therapy. [References: 42]

Go to ... Help | Logoff

Citation 4

Authors

Bosse P. Bernex F. Desepulveda P. Salaun P. Panthier JJ.

Title

MULTIPLE NEUROENDOCRINE TUMOURS IN **TRANSGENIC** MICE INDUCED BY C-KIT-SV40 T ANTIGEN FUSION GENES

Source

Oncogene. 14(22):2661-2670, 1997 Jun 5.

Abstract

Transgenic mice carrying either a 1.008 or a 4.225 kb of the mouse c-kit 5'-flanking sequences linked to the oncogenic large T antigen (TAg) region of the sinian virus 40 (SV40) genome were generated to test if the c-kit promoter could be used to develop useful mouse models. Both constructs promote tumourigenesis in the pituitary and the thyroid with high efficiency. The cell types from which each of these tumours derives were identified. Tumours of the pituitary derive from alpha-MSH-expressing cells located in the intermediate lobe, Transformed cells of the thyroid were calcitonin-positive, implying that the tumours derive from C cells or their precursors, Chromogranin A and **neuron-specific enolase**, general neuroendocrine cell markers, were expressed in both tumour types. Furthermore a variety of tumours appeared in the **transgenic** mice. Several of them stained positively for chromogranin A and/or **neuron-specific enolase**, This suggests a previously unsuspected tissue-**specific**ity of the c-kit 5' flanking sequences for neuroendocrine cells. The Kit-TAg **transgenic** mouse Lines may represent a valuable model for the study of the development and the biology of neuroendocrine tumours. [References: 53]

Go to ... Help | Logoff

Citation 5

Authors

Twyman RM. Jones EA.

Title

SEQUENCES IN THE PROXIMAL 5' FLANKING REGION OF THE RAT **NEURON-SPECIFIC ENOLASE** (NSE) GENE ARE SUFFICIENT FOR CELL TYPE-**SPECIFIC** REPORTER GENE EXPRESSION

Source

Journal of Molecular Neuroscience. 8(1):63-73, 1997 Feb.

Abstract

We investigated the regulation of the rat **neuron-specific enolase** gene using a transient transfection approach. Recent **transgenic** mouse studies have shown that a 1.8-kb segment of the rat NSE gene 5' flanking region, including the first (noncoding) exon but not the first intron, is able to drive expression of a reporter gene in parallel with endogenous NSE. These data suggest that cis-acting elements responsible for the spatial and temporal pattern of NSE gene expression are located within the proximal 1.8 kb of the 5' flanking sequence. To further investigate this region, we joined the 1.8-kb regulatory cassette to the cat reporter gene and generated a number of constructs in which the flanking sequence was

progressively deleted from the 5' end. These constructs were tested by transient transfection into **neuron**al and non**neuron**al cells, followed by an assay for CAT activity. We found that as little as 255 bp of 5' flanking sequence was able to confer cell type-**specific**ity on the reporter gene. Further truncation to 120 bp of 5' sequence resulted in a sharp downregulation of reporter activity in PC12 cells but a significant rise in both Neuro-2A neuroblastoma cells and non**neuron**al Ltk- cells, indicating that cis-acting elements controlling the regulation of NSE in Ltk-, Neuro-2A, and PC12 cells may lie within the 135 bp region covered by this deletion. This region contains an AP-2 site and an element similar in sequence and position to a motif identified in the proximal promoter region of the **neuron-specific** peripherin gene. Reduction to 95 bp of 5' sequence resulted in a slight downregulation of CAT activity in all cell lines tested, and further truncation to 65 bp of 5' sequence caused a universal reduction to background levels of CAT activity, concomitant with the disruption of the basal NSE promoter. Our results show that the 5' flanking region of the NSE gene is capable of conferring cell type-**specific**ity on a heterologous gene in transfected cells and that elements responsible for this are located within the proximal 255 bp. [References: 33]

Go to ... Help | Logoff

Citation 6

Authors

Coluccidamato GL. Santelli G. Dalessio A. Chiappetta G. Mineo A. Manzo G. Vecchio G. Defranciscis V.

Title

DBL EXPRESSION DRIVEN BY THE **NEURON SPECIFIC ENOLASE** PROMOTER INDUCES TUMOR FORMATION IN **TRANSGENIC** MICE WITH A P53(+/-) GENETIC BACKGROUND

Source

Biochemical & Biophysical Research Communications. 216(3):762-770, 1995 Nov 22.

Abstract

The dbl oncogene, generated by the truncation of the amino-terminal portion of the proto-oncogene sequence, encodes a guanine-nucleotide-releasing factor. The transforming activity of this oncogene has never been demonstrated in vivo or in vitro except in the NIH 3T3 mouse fibroblast cell line. The expression of the proto-dbl transcript is confined to tissues and tumors of neuroectodermal derivation. Therefore, to study the transforming activity of the dbl oncogene in vivo. we have generated **transgenic** mice that express this oncogene in neuroepithelial tissues. Mice carrying the dbl oncogene did not develop a tumor. Successively, to establish whether dbl interacts with the tumor suppressor gene p53 in tumorigenesis, we have used a p53 deficient mouse strain. The results reported here indicate that dbl is capable of causing tumor formation in vivo when its expression is driven in an appropriate cellular and genetic environment. (C) 1995 Academic Press, Inc. [References: 19]

Go to ... Help | Logoff

Citation 7

Authors

Race RE. Priola SA. Bessen RA. Ernst D. Dockter J. Rall GF. Mucke L. Chesebro B. Oldstone MBA.

Title

NEURON-SPECIFIC EXPRESSION OF A HAMSTER PRION PROTEIN MINIGENE IN **TRANSGENIC** MICE INDUCES SUSCEPTIBILITY TO HAMSTER SCRAPIE AGENT

Source

Neuron. 15(5):1183-1191, 1995 Nov.

Abstract

To study the effect of cell type-restricted hamster PrP expression on susceptibility to the hamster scrapie agent, we generated **transgenic** mice using al kb hamster cDNA clone containing the 0.76 kb HPrP open reading frame under control of the **neuron-specific enolase** promoter. In these mice, expression of HPrP was detected only in brain tissue, with highest levels found in **neuron**s of the cerebellum, hippocampus, thalamus, and cerebral cortex. These **transgenic** mice were susceptible to infection by the 263K strain of hamster scrapie with an average incubation period of 93 days, compared to 72 days in normal hamsters. In contrast, non**transgenic** mice were not susceptible to this agent. These results indicate that **neuron-specific** expression of the 1 kb HPrP minigene including the HPrP open-reading frame is sufficient to mediate susceptibility to hamster scrapie, and that HPrP expression in non**neuron**al brain cells is not necessary to overcome the TSE species barrier. [References: 61]

Go to ... Help | Logoff

Citation 8

Authors
Alouani S. Ketchum S. Rambosson C. Eistetter HR.

Title
TRANSCRIPTIONAL ACTIVITY OF THE
NEURON-SPECIFIC ENOLASE
(NSE) PROMOTER IN MURINE EMBRYONIC STEM (ES) CELLS AND PREIMPLANTATION EMBRYOS

Source
European Journal of Cell Biology. 62(2):324-332, 1993 Dec.

Abstract
Mouse embryonic stem (ES) cells were transfected with a plasmid composed of an E. coli lacZ gene fused to 1.8 kb of rat **neuron-specific enolase** (NSE) promoter sequences. While this reporter construct had been shown previously to function exclusively in postmitotic **neuron**s and neuro-endocrine cells of **transgenic** mice, stably transfected ES cell clones unexpectedly displayed beta-galactosidase (beta-Gal) activity in the undifferentiated state. This transcriptional activity of the heterologous NSE promoter was confirmed by the identification of endogenous NSE mRNA in undifferentiated ES cells, mouse morulae and blastocysts. NSE protein, however, could not be found in undifferentiated ES cells. Interestingly, in ES cells which were cultured for 7 days under differentiation conditions in vitro, beta-Gal activity decreased to basal levels consistent with the parallel down-regulation of endogenous NSE mRNA. In contrast, prolonged culture of ES cells under differentiation conditions led to the reappearance of NSE mRNA and beta-Gal activity after 17 days. Significant increases in beta-Gal activity were also observed in ES cells which were cultured either on dishes coated with attachment factors such as laminin and gelatin or in the presence of nerve growth factor (NGF). These results suggest that i) transcriptional control mechanisms regulating **neuron**al gene expression are present at early developmental stages in the mouse and ii) ES cells provide a useful in vitro model system for the analysis of developmentally regulated cellular and molecular events coupled to **neuron-specific enolase** promoter activity.
[References: 42]

Go to ... Help | Logoff

Citation 9

Authors
Andersen JK. Frim DM. Isacson O. Breakefield XO.

Title
HERPESVIRUS-MEDIATED GENE DELIVERY INTO THE RAT BRAIN -
SPECIFICITY AND EFFICIENCY OF THE
NEURON-SPECIFIC ENOLASE
PROMOTER

Source
Cellular & Molecular Neurobiology. 13(5):503-515, 1993 Oct.

Abstract
1. Herpesvirus infection with genetically engineered vectors is a way to deliver foreign gene products to various cell populations in culture and in vivo. Selective **neuron**al gene expression can be achieved using the **neuron-specific enolase** (NSE) promoter regulating expression of a transgene placed in and delivered by a herpesvirus vector. 2. We sought to determine the anatomical **specific**ity and efficiency of herpesvirus-mediated gene transfer into the rat brain following placement of virus particles carrying a transgene (lacZ) under control of the NSE promoter. The virus utilized was thymidine kinase (TK) deficient and therefore replication deficient in the brain. 3. Infusion of 10(6) plaque-forming units of virus into the striatum caused a limited number of striatal **neuron**s to express the lacZ transgene mRNA and protein product 7 days postinfection. In addition, small numbers of **neuron**s expressing the transgene mRNA and protein were found ipsilateral to the viral injection in the frontal cortex, substantia nigra pars compacta, and thalamus. **Neuron**s at these anatomic loci project directly to the striatal injection site. No other cells within the brains of injected animals expressed the lacZ gene. 4. While this herpesvirus NSE vector was capable of introducing novel functional genetic information into postmitotic **neuron**s within defined neuroanatomic constraints, the numbers of **neuron**s expressing detectable levels of beta-galactosidase was minimal. The calculated efficiency of delivery and transgene expression at 7 days postinfection was 1 **transgenic neuron** per 10(4) virus particles infused. 5. We conclude that NSE probably is not an optimal promoter for use in gene delivery to CNS **neuron**s in herpesvirus vectors and that the efficacy of gene delivery using other **neuron-specific** promoters placed at various sites in the herpes viral genome needs to be explored.
[References: 47]

Main Search Page

Heparan sulfate: a piece of information

MARKKU SALMIVIRTA, KERSTIN LIDHOLT, AND ULF LINDAHL[1]

Department of Medical and Physiological Chemistry, Uppsala University, S-75123 Uppsala, Sweden

ABSTRACT **The sulfated glycosaminoglycans, heparan sulfate and heparin, are increasingly implicated in cell-biological processes such as cytokine action, cell adhesion, and regulation of enzymic catalysis. These activities generally depend on interactions of the polysaccharides with proteins, mediated by distinct saccharide sequences, and expressed at various levels of specificity, selectivity, and molecular organization. The formation of heparin/heparan sulfate in the cell requires an elaborate biosynthetic machinery, that is conceived in terms of a novel model of glycosaminoglycan assembly and processive modification. Recent advances in the identification and molecular analysis of the enzymes and other proteins involved in the biosynthesis provide novel tools to study the regulation of the process, presently poorly understood, at the subcellular and cellular levels. The potential medical importance of heparin-related compounds is likely to promote the biotechnological exploitation of components of the biosynthetic machinery.—Salmivirta, M., Lidholt, K., Lindahl, U. Heparan sulfate: a piece of information. *FASEB J.* 10, 1270–1279 (1996)**

Key Words: heparin · GAG · proteoglycan · enzyme · polysaccharide-protein interactions

HEPARIN, A MAMMALIAN glycosaminoglycan (GAG),[2] has the highest negative charge density of any known biological macromolecule. It thus is prone to ionic interaction with a variety of proteins such as enzymes, enzyme inhibitors, extracellular-matrix proteins, various cytokines, and others (1). Such interaction is exploited in the purification of "heparin-binding proteins," which are adsorbed to immobilized heparin at low ionic strength and subsequently eluted with salt. The appreciable purification often achieved suggests an element of selectivity beyond that expected for simple cation-exchange chromatography.

Heparin is isolated on a commercial basis from animal tissues (pig intestinal mucosa; bovine lung) and is used in the clinic as an antithrombotic drug. In the intact tissue it is confined to mast cells, where it is stored in cytoplasmic granules. Heparan sulfate (HS), on the other hand, has ubiquitous distribution on cell surfaces and in the extracellular matrix. It is generally less sulfated than heparin and has a more varied structure. Interactions between HS and specified proteins are being increasingly implicated in a variety of physiological processes, such as cell adhesion, enzyme regulation, cytokine action, etc. (1).

Heparin and HS are both synthesized as proteoglycans (PGs), which consist of GAG chains covalently bound to a protein core. A single protein, serglycin, has been identified as the protein constituent of heparin PGs, whereas a variety of proteins provide core structures of HS PGs (2–4). Biosynthesis of either heparin or HS PGs involves the formation of an initial, simple GAG structure, composed of alternating D-glucuronic (GlcA) and N-acetyl-D-glucosamine (GlcNAc) units, joined by 1 → 4 linkages (**Fig. 1*A***). This structure may then be modified through a series of reactions that ultimately result in the formation of -IdoA(2-OSO_3)-GlcNSO_3(6-OSO_3)- sequences [where L-iduronic acid (IdoA) is the C5-epimerization product of GlcA] (Fig. 1*B*). This process, which generates the most abundant disaccharide unit in heparin, will be referred as heparin "default modification."

Heparin, and in particular, HS, contain structures that deviate from the product of default modification. Such structures arise through "modulated modification," which may differ from the default process in either of two ways. The pathway may be incomplete, due to lack of all (Fig. 1*A*) or some of the reactions, or it may involve additional reactions. Some structures generated through modulated polymer modification are shown in Fig. 1*C–F*.

POLYSACCHARIDE-PROTEIN INTERACTIONS

Binding of heparin/HS sequences to proteins is generally (although not exclusively) ionic, and thus involves positively charged, usually clustered, amino acid residues in the protein components. Attempts to define polypeptide consensus sequences for heparin binding have yielded partly contradictory results (5). Conversely, the anionic

[1]To whom correspondence and reprint requests should be addressed, at: Department of Medical and Physiological Chemistry, Uppsala University, Biomedical Center, P.O. Box 575, S-75123 Uppsala, Sweden.

[2]Abbreviations: FGF, fibroblast growth factor; GAG, glycosaminoglycan; GalNAc, 2-acetamido-2-deoxy-D-galactose (N-acetylgalactosamine); GlcNAc, 2-acetamido-2-deoxy-D-glucose (N-acetylglucosamine); $GlcNH_3^+$, N-unsubstituted glucosamine; GlcA, D-glucuronic acid; HS, heparan sulfate; IdoA, L-iduronic acid; PAPS, 3′-phosphoadenosine-5′-phosphosulfate; PG, proteoglycan.

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protein binding domains of polysaccharide chains may differ with regard to structure, degree of binding specificity, and organization at the macromolecular level (**Fig. 2**). Simple interactions involve single binding sites on the GAG chain (Fig. 2*A*) and on the protein moiety. Ternary complexes may contain two (identical or distinct) proteins bound to separate domains on the same GAG chain (Fig. 2*B*). Finally, different protein binding domains may be located on separate GAG chains that are bound to a common core protein in a PG (Fig. 2*C*).

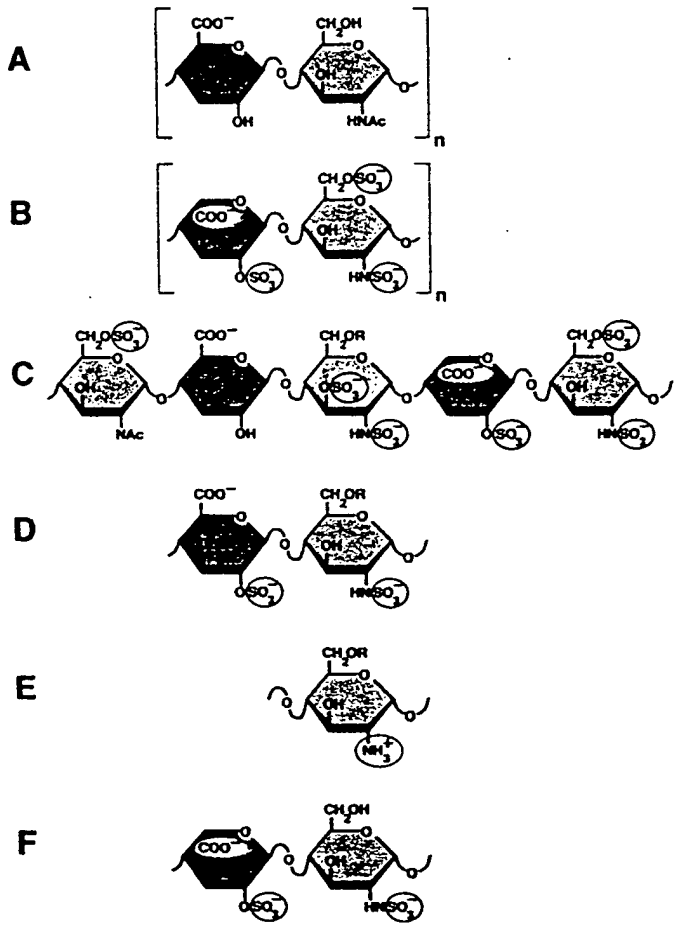


Figure 1. Examples of heparin/HS structures produced by "default" and "modulated" polymer modification. The initial polymerization product is a repeat of alternating GlcA and GlcNAc units (*A*). Approximately 80% of such disaccharides in heparin, but generally < 10% in HS, undergo default modification involving N-deacetylation and N-sulfation of GlcN-units, C-5 epimerization of GlcA to IdoA, and O-sulfation at two positions, yielding the -IdoA(2-OSO_3)-GlcNSO_3(6-OSO_3)- disaccharide unit (*B*). Products of modulated modification include the "unique" 3-O-sulfated GlcNSO_3 unit in the middle of the antithrombin binding pentasaccharide sequence (*C*). The -GlcA(2-OSO_3)-GlcNSO_3- disaccharide unit (*D*) is present in different HS species in highly variable amounts. A small proportion of GlcN residues in heparin and HS contain unsubstituted amino groups (*E*). The -IdoA(2-OSO_3)-GlcNSO_3- unit (*F*) is a common product of modulated (restricted) modification typical for HS. Hexuronic acid residues are shown in blue, glucosamine units are green, and sulfate groups yellow. For further information, see the text.

Protein binding regions generated by default modification

Interactions between heparin/HS and proteins generally depend on the presence of sulfate groups. Although this requirement is readily demonstrated by using chemically desulfated GAG preparations, it is more difficult to pinpoint those sulfate groups that are actually essential for binding. Saccharide sequences composed of repeating, default-modified disaccharide units, -[IdoA(2-OSO_3)-GlcNSO_3(6-OSO_3)]$_n$-, abundant in heparin and frequently found within the N-sulfated block regions of HS chains (6), bind to many proteins. Still, the precise requirement for individual sulfate groups within these sequences may vary from one "heparin binding" protein to another.

The enzyme lipoprotein lipase binds to HS chains of PGs at the surface of vascular endothelial cells, with marked preference for a saccharide sequence consisting exclusively of the trisulfated disaccharide unit (7). Other proteins, such as thrombin (8) and platelet factor 4 (9), bind to the same sequence in seemingly nonspecific fashion. The problem of specificity was highlighted in a series of studies involving members of the fibroblast growth factor (FGF) family. These proteins all bind heparin, apparently via the same sequence of default-modified (trisulfated) disaccharide units. However, attempts to define the minimal binding sequence for FGF-2 (basic FGF) revealed a pentasaccharide structure in which the essential sulfate groups were limited to a single IdoA 2-O-sulfate and one or two N-sulfate groups (10, 11). The remaining [IdoA(2-OSO_3) and GlcN(6-OSO_3)] O-sulfate groups of the fully sulfated heparin structure would seem not to contribute to or interfere with FGF-2 binding.

Protein binding regions generated by modulated modification

A concept of protein binding to the default-modified heparin sequence, but with selective involvement of sulfate groups, has intriguing implications. Indeed, the available data suggest that binding of different members of the FGF family may require different combinations of sulfate groups, hence different saccharide sequences (5, 12). Such sequences may well all be represented, albeit in "hidden" form, by the same fully sulfated, default-modified heparin structure shown in Fig. 1*B*, given the proviso that sulfate groups other than those of the implicated minimal sequences will not interfere with protein binding. Conceivably, however, they may also be differentially expressed in separate GAG chains of the HS type (**Fig. 3**). The generation of such specific saccharide ligands would require selective restriction of polymer modification. Consequently, minimal sequences are more likely to occur in HS than in heparin due to the more variable, and generally lower, degree of modification of the former species.

Modulated polymer modification may also result in the formation of rare ("unique") structural components that

are introduced through distinct reactions. An example of such a marker component that is implicated with a defined biological function is the 3-O-sulfated GlcN unit, which is located in the antithrombin binding pentasaccharide sequence of heparin and HS (Fig. 1*C*) and is essential to the blood anticoagulant activity of the polysaccharides (reviewed in ref 8). Another generally minor and variable component is the 2-O-sulfated GlcA unit (Fig. 1*D*), which contributes to the structural distinction between HS species from different sources. A -GlcA(2-OSO_3)-GlcNSO_3- disaccharide unit thus accounts for as much as 11% of the total N-sulfated disaccharide units of HS from adult human cerebral cortex, but is virtually absent in the corresponding neonatal material as well as in HS preparations from other adult tissues, such as the arterial wall (13). These findings suggest an organ-specific and age-related control of GlcA 2-O-sulfation. Even though the precise functional role of the sulfated GlcA residue is unknown, previous studies of cultured hepatocytes revealed a nuclear pool of HS with a strikingly high content of the -GlcA(2-OSO_3)-GlcNSO_3(6-OSO_3)- disaccharide unit (14). Nuclear HS was tentatively implicated with the control of cell proliferation. Heparin [which also contains small amounts of GlcA(2-OSO_3) units (2)] is known to bind the transcription factors Fos and Jun and to inhibit their effects on gene expression (15).

A small proportion of GlcN residues in heparin and HS preparations have unsubstituted amino groups (Fig. 1*E*). Recent immunohistochemical work established that the N-unsubstituted GlcN (GlcNH_3^+) units occur in native HS and are not due to preparation artifacts (as was tacitly assumed in the past) (16). Moreover, an antibody recognizing GlcNH_3^+-containing epitopes stained HS in glomerular basement membranes, but not that in tubular basement membranes of the rat kidney, suggesting a selective expression of the epitope. A possible clue to the functional role of this structure emerged through the finding that L-selectin preferentially bound to GlcNH_3^+-containing HS PGs (17). However, chemical N-acetylation of such PG did not impede L-selectin binding. It was proposed that the presence of a GlcNH_3^+ unit might regulate the biosynthetic modification of surrounding saccharide sequences, thus creating a structure recognized by L-selectin.

Multiple interaction sites

HS modulates the biological activity of interferon-γ by interacting with the dimeric cytokine. Interferon binding HS fragments, encompassing as many as 40–50 monosaccharide units, were shown to consist of two terminal sulfated domains, each binding to one interferon-γ monomer, separated by a nonsulfated GlcA-rich sequence (18). This finding points to an important general concept: properly spaced sequences of the appropriate structure along a GAG chain may form functional domains that act in a concerted manner (Fig. 2*B*). Such domains may bind to identical peptide sites, as in the case of interferon-γ, or to different sites, as in the heparin-antithrombin-thrombin interaction (8). Whereas antithrombin binds to a specific pentasaccharide sequence (see above), a much longer saccharide (minimal size ~18 monosaccharide units) is required to induce thrombin inhibition. This observation reflects the requirement for a ternary complex in which not only antithrombin but also thrombin bind to the polysaccharide chain (19).

An analogous mode of interactions has been proposed for the binding of heparin/HS to FGF-2 and its cell-surface tyrosine kinase type receptor (20). The minimal heparin/HS sequence required to promote FGF-2-induced cell proliferation consists of ~12 monosaccharide units, more than twice the size of the pentasaccharide region that actually binds the growth factor (see above). One possible explanation to this finding is that binding of two growth factor molecules to adjacent sites on the GAG chain will promote receptor dimerization, as required for receptor activation (21). However, there is evidence for

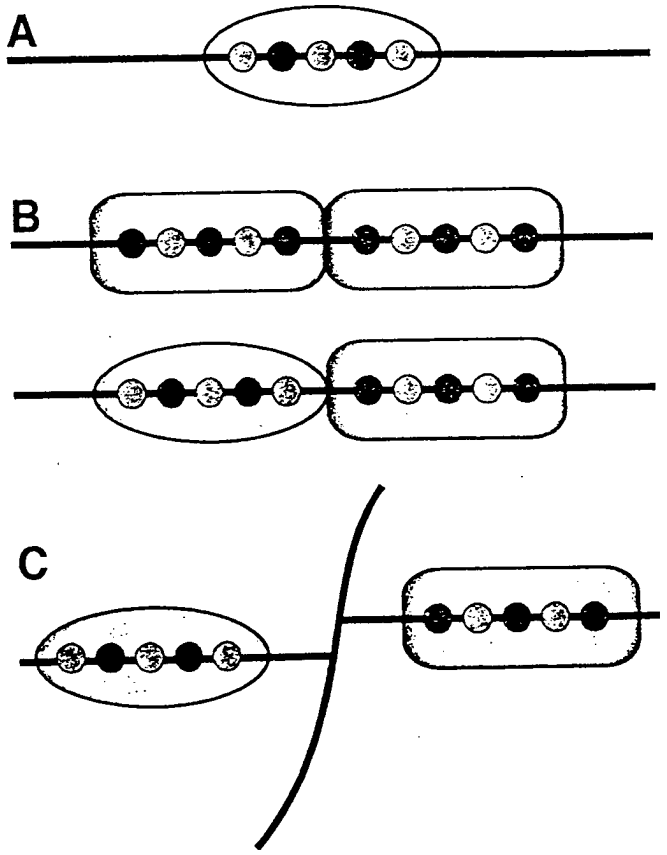


Figure 2. Models of differentially organized single and multiple protein binding domains in heparin/HS. Single protein binding sites along a polysaccharide chain (*A*) may vary in size and composition depending on ligand specificity. Two similar sequences located adjacent to each other (upper model in panel *B*) may form a composite binding domain for two identical ligands, such as the subunits of a protein dimer. An analogous arrangement of two nonidentical domains will promote the formation of ternary complexes between the saccharide chain and two distinct proteins (lower model in panel *B*). Finally, similar or distinct, single or multiple binding domains may reside in separate HS chains of a PG (*C*), thus providing a functional versatility for a PG molecule that is not possessed by its individual HS chains. For further information, see the text.

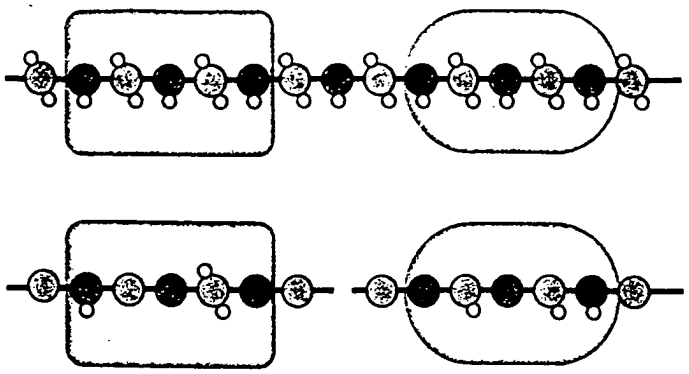


Figure 3. Hidden specificity in protein binding. Heparin is able to bind a large number of proteins via its default-modified disaccharide repeats, as illustrated by the binding of two distinct proteins to apparently similar heparin structures (upper complexes). However, the identification of minimal protein binding domains in HS suggests that only some of the sulfate groups present in the default-modified sequence will actually participate in a given interaction (lower complexes). Contrary to the default-modified sequence, such minimal sequences are protein-selective, such that the HS sequence in the lower left complex is unable to bind the protein on the right. For further information, see the text.

direct binding of heparin to the receptor itself (22); indeed, FGF receptor 4 can be activated by heparin, even in the absence of growth factor (23). The effects of selectively O-desulfated heparin preparations in FGF-2-dependent cell proliferation assays suggested that both IdoA 2-O- and GlcN 6-O-sulfate groups were needed for activity (20), contrary to growth factor binding alone, which required 2-O-sulfate groups only (see above). These and other (24) findings were interpreted in terms of a ternary complex, with FGF-2 and its receptor binding to adjacent, distinct sites on the same polysaccharide chain. This model predicts that whereas a GAG chain that contains the appropriately spaced growth factor binding and receptor binding sequences will promote the FGF-induced cellular response, other species with only one of the sequences, or with incorrectly spaced binding regions, will inhibit the response. Domain spacing, in turn, is determined by the length of any intervening sequence and further influenced by the conformational flexibility of such sequences.

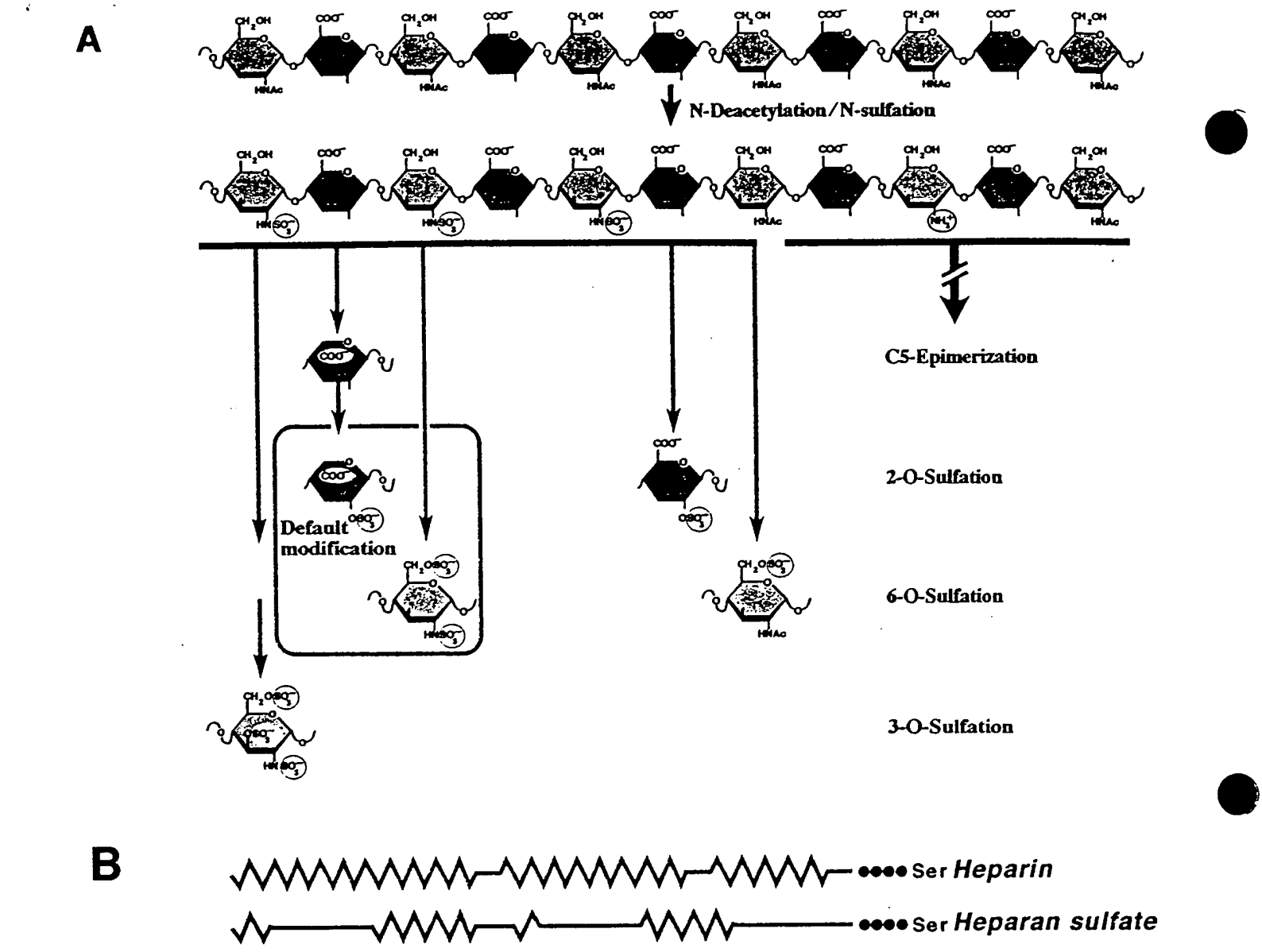
Finally, we consider the possibility of different functional GAG domains residing in different HS chains that are bound to the same core protein (Fig. 2*C*). PGs differ from each other both in the number of potential HS attachment sites and the location of such sites along the protein backbone (2, 3). In glypican, for example, the HS attachment sites appear to be predominantly located close to each other, between the membrane-bound domain and a large extracellular globular domain, whereas syndecans 1 and 3 have more extended core proteins that may carry HS both at the proximal and distal ends of their extracellular domains. The latter arrangement could conceivably facilitate the differential interaction of separate HS chains with several proteins concomitantly. Indeed, syndecan-1, immobilized by interacting through one of its HS chains with fibronectin or collagen, retains its ability to bind FGF-2. By contrast, free HS chains derived from syndecan-1 can bind to only one of the two proteins at a time (25), suggesting a functional versatility for intact PG that is not expressed by the individual HS chain.

POLYSACCHARIDE BIOSYNTHESIS

The biosynthesis of heparin and HS and the regulatory mechanisms required to generate different saccharide sequences of defined structure are only partly understood. Lacking any "code" that specifies such sequences, we need to characterize in detail the enzymes that catalyze the assembly of GAG chains, their concerted mode of action, and their subcellular organization. This discussion will focus on the recent development of the area; for references to older work, see ref 1.

Polysaccharide chain initiation

The enzymes responsible for GAG biosynthesis are located largely in the Golgi apparatus. A tetrasaccharide "linkage region" (-glucuronic acid-galactose-galactose-xylose-) attached to a serine residue in a core protein provides the starting point for polysaccharide chain elongation. The same linkage region is found in PGs carrying glucosaminoglycan (heparin/HS) or galactosaminoglycan (chondroitin sulfate/dermatan sulfate) chains, and there are indications that the same enzymes catalyze the formation of this region in the different types of PGs (26). Once formed, the linkage region will serve as acceptor for the first GlcNAc or GalNAc unit in a reaction that will commit the process toward generation of a glucosamino- or a galactosaminoglycan chain. The hexosaminyl-transferases that add the first GlcNAc/GalNAc units to the linkage region appear to differ from those involved in actual chain elongation, but the factors that determine whether a GlcNAc or a GalNAc unit is to be added remain unclear. Peptide sequence motifs close to GAG-substituted serine residues have been implicated as a signal for the addition of a GlcNAc unit to the linkage tetrasaccharide, thus initiating heparin/HS formation (ref 27 and references therein); an α-GlcNAc transferase catalyzing this reaction has been demonstrated (28). The absence of such a signal would lead to "default" substitution of the linkage region with a GalNAc unit, followed by chondroitin formation. Unexpectedly, transfer of a GalNAc residue to the same tetrasaccharide structure, catalyzed by enzymes present either in bovine fetal serum (29) or in mouse mastocytoma tissue (K. Lidholt, M. Fjelstad, U. Lindahl, T. Ogawa and K. Sugahara, unpublished results), resulted in the incorporation of the α-anomeric sugar rather than the β-GalNAc unit occurring in this position in native chondroitin or dermatan sulfate. Although the significance of this finding is unclear, it is conceivable that the α-GalNAc unit may serve as a general stop signal that prevents further GAG formation. Likewise, the role of the sulfate substituents located at the galactose



residues linked to chondroitin and dermatan sulfate chains, but lacking in those attached to heparin and HS, is not understood (see ref 29 for references).

Formation of the polysaccharide chain

After completion of the tetrasaccharide linkage region, the GAG chain proper is formed by alternating transfer of GlcA and GlcNAc monosaccharide units from the corresponding UDP-sugar nucleotides to the nonreducing termini of nascent chains. This process can be demonstrated in cell-free systems (microsomal preparations) in the absence of subsequent polymer-modification reactions. However, chain elongation is promoted by concomitant N-sulfation, and it is currently believed that, in the intact cell, this and other modification reactions (see below) occur while the chain is still being elongated (30) (**Fig. 4C** and **Fig. 5**).

Modification of the $(GlcA\text{-}GlcNAc)_n$ polymer is initiated by N-deacetylation and N-sulfation of GlcNAc units, the latter step as well as the subsequent sulfotransferase reactions requiring 3′-phosphoadenosine-5′-phosphosulfate (PAPS) as a sulfate donor. The pathway previously referred to as "default modification" further involves C5-epimerization of GlcA to IdoA units, which are then O-sulfated at C2, and finally O-sulfation of $GlcNSO_3$ units at C6 (Fig. 4). Deviations from this pathway may be due to either restricted or further extended modification. Reactions of the latter category, which generally involve only minor portions of the polysaccharide chains, include O-sulfation at C2 of GlcA (31) and at C3 of GlcN units (Fig. 4; for references, see ref 1).

The most conspicuous restriction of polymer modification is due to incomplete N-deacetylation/N-sulfation. Because the enzymes that catalyze the C5-epimerization and various O-sulfation reactions all require N-sulfate groups for substrate recognition (within a defined distance from the actual target site), sequences composed of consecutive N-acetylated disaccharide units will be devoid of IdoA and O-sulfate residues (1, 6). Such sequences (blue in Fig. 4) are typical for HS but are rare in heparin chains (1). Given the constraints of the modification

C

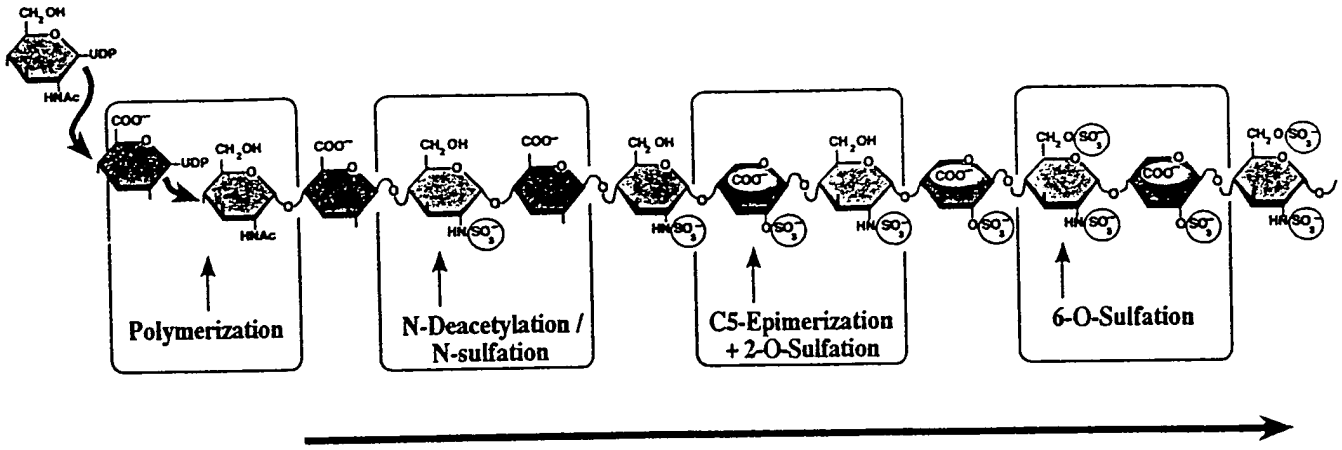


Figure 4. *A)* Polymer-modification reactions involved in the biosynthesis of heparin and HS. The first modification step, N-deacetylation and N-sulfation of GlcN-units, has a key regulatory function because the regions that remain N-acetylated (underlined in blue) will largely escape subsequent modification steps as well (note that an N-acetylated GlcN unit may become 6-O-sulfated, provided that one of the adjacent GlcN residues is N-sulfated). By contrast, N-sulfated regions (underlined in red) are subject to further modification, involving either the default pathway (boxed), a more restricted process (e.g., leaving a nonsulfated IdoA unit), or more extensive modification (e.g., 3-O-sulfation of a $GlcNSO_3$ unit). The position (or positions) of N-unsubstituted GlcN residues (arbitrarily allocated to the N-acetylated region) have not yet been defined. *B)* Scheme showing the domain organization of heparin and HS. The heparin polymer consists of extended, highly modified domains occasionally interspersed by short unmodified domains. HS, on the other hand, typically features unmodified domains of variable length (that may constitute ≥50% of the total polymer) alternating with heterogeneous N-sulfated domains. The four black circles represent the GlcA-Gal-Gal-Xyl linkage region that is bound to a serine residue in the protein core. *C)* Proposed course of polymer formation and default modification in heparin/HS biosynthesis. The indicated coupling between the C5-epimerization and 2-O-sulfation reactions applies to the default modification pathway, but is not mandatory. The direction of passage of the elongating polymer through the modification machinery is indicated by the red arrow, the resultant default-modified chain thus exiting the putative enzyme complex after 6-O-sulfation of $GlcNSO_3$ units. For further information, see the text.

process dictated by substrate specificity and access to a survey of authentic identified structures, a scheme of "permitted" and "forbidden" sequences has been compiled that presumably applies to the entire heparin/HS family (Fig. 2 in ref 1). This scheme does not include the N-unsubstituted GlcN unit, which has yet to be placed in a structural context (see legend to Fig. 4).

Regulation of polymer modification: implications of a model

The scheme in Fig. 4 illustrates the assumed order of the various modification reactions, as deduced essentially from the substrate specificities of the corresponding enzymes. However, the mode of selection of target residues extends beyond a simple matter of substrate specificity. We have no clue as to what mechanism determines whether a particular region of a precursor polysaccharide is going to be N-deacetylated/N-sulfated, and thus subject to further modification, or remain N-acetylated and unmodified. Even within the N-sulfated regions (red in Fig. 4), potential target units often escape modification. For example, a GlcA unit located between two N-sulfated GlcN residues may undergo C5-epimerization to IdoA, but may also remain unchanged. Similarly, 6-O-sulfation of a $GlcNSO_3$ unit located between two IdoA(2-OSO_3) residues is optional. Due to such selectivity, the structural complexity and heterogeneity of the polysaccharide chain will increase through the modification process. The functional relevance of the selection mechanism is apparent, as it provides the basis for the generation of protein binding regions of defined structure.

Our current model depicting the physical course of heparin/HS formation (Fig. 5) features simultaneous elongation and modification of the polysaccharide precursor, in accord with the postulated coupling between the polymerization and N-deacetylation/N-sulfation reactions (30). A glycosyltransferase/N-deacetylase/N-sulfotransferase complex, located at the nonreducing end of the chain, will generate saccharide sequences with N-sulfate or residual N-acetyl groups, which are then subjected to further downstream modification by enzymes that act in a processive fashion along the polysaccharide chain.

Given the main features of this model, we may consider the generation of some structural domains identified in heparin/HS chains. A process in which every disaccharide unit formed would be attacked by each enzyme indicated in Fig. 5 (except the 3-O-sulfotransferase) would lead to a uniform product with the structure expected from default modification (Fig. 1*B*). Deviations from this course involving restricted modification, the hallmark of HS biosynthesis, would require an interrupted, on-off

mode of processive enzyme action. The versatility of this modulation is intriguing. How can we visualize the generation, through the action of a common assembly line, of a polysaccharide chain that contains extended N-acetylated as well as N-sulfated regions of varying length, but also sequences of alternating N-acetylated and N-sulfated disaccharide units that may account for as much as 30% of the total mass of a HS chain (6). By what mechanism (or mechanisms) are certain disaccharide units within a contiguous N-sulfated block sequence selected to escape the processive (?) action of one or more of the three major "downstream" enzymes, i.e., the GlcA C5-epimerase, the IdoA 2-O-sulfotransferase, and the GlcN 6-O-sulfotransferase? What causes the apparent separation of

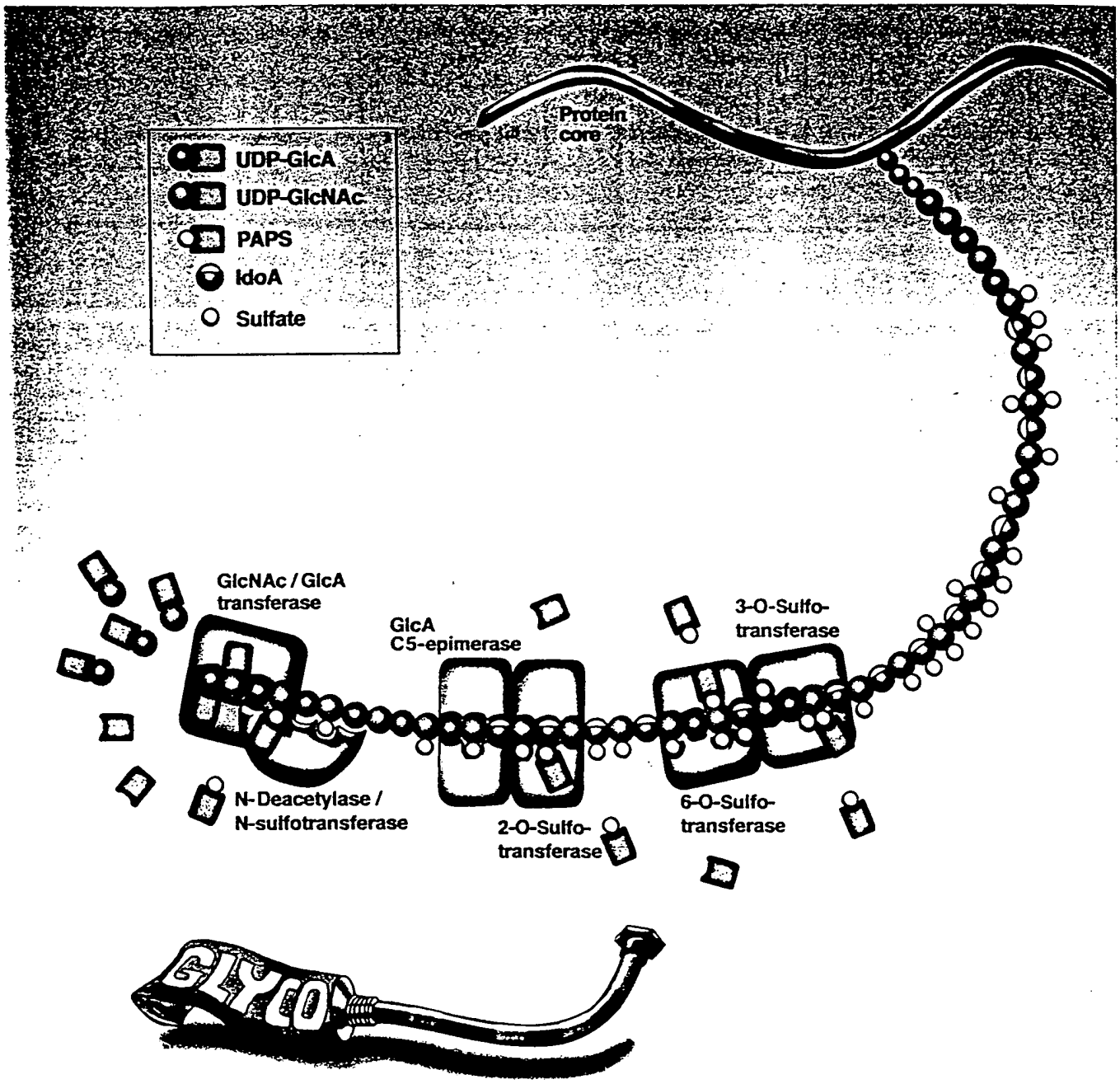


Figure 5. A model of the biosynthetic assembly of heparin/HS in the Golgi complex. A nascent polysaccharide chain, the reducing end bound to a protein core, is shown to traverse the components of the biosynthetic enzyme machinery. The building blocks of the chain, i.e., sugar nucleotides and PAPS, are transported across the Golgi membrane from the cytoplasm where their formation is fueled by cellular ATP. The "polymerase" (GlcNAc/GlcA transferase) adds alternating GlcNAc and GlcA units to the elongating polymer, which is concomitantly, yet sequentially, modified by enzymes catylyzing N-deacetylation and N-sulfation of GlcNAc units, C5-epimerization of GlcA to IdoA units, and O-sulfation at various positions. Enzymes (the "polymerase" and the GlcNAc N-deacetylase/N-sulfotransferase) so far shown to catalyze more than one reaction are indicated. The remaining enzymes have been arbitrarily combined into complexes; indeed, the organization of all enzymes into one major complex appears entirely plausible. The mode of interaction of such a complex with the polysaccharide chain, resulting in the generation of alternating modified (red in Fig. 4) and unmodified (blue in Fig. 4) sequences, is not understood. For further information, see the text. Modified from ref 30.

target sites for 2-O-sulfation, essentially restricted to N-sulfated blocks, and 6-O-sulfation, which occurs within as well as outside these blocks (32; 32a).

The formation of a "unique" marker component is more tangible insofar as it may require the participation of a distinct enzyme. The biosynthesis of the antithrombin binding region in heparin and HS thus is concluded by a GlcN 3-O-sulfotransferase (Figs. 4 and 5) that recognizes as sulfate acceptor a pentasaccharide sequence that differs from the functional binding site (Fig. 1*C*) only by lacking the GlcN 3-O-sulfate target residue (see ref 33). Formation of the acceptor sequence again depends on restricted polymer modification, because the single GlcA unit is essential for substrate recognition. However, analysis of polysaccharide chains lacking the 3-O-sulfate group (hence with low affinity for antithrombin and low blood anticoagulant activity) showed the occurrence of potential 3-O-sulfate acceptor sites that apparently had escaped attack by the enzyme (33). Thus, the GlcN 3-O-sulfotransferase, similar to the IdoA 2-O- and the GlcN 6-O-sulfotransferases, is also subject to regulation based on restricted access to substrate sequences. Overexpression in endothelial cells of a specific HS PG core protein (syndecan-4) that normally carries 3-O-sulfated HS chains led to selective depression of 3-O-sulfation, hence to impeded formation of HS chains with high affinity for antithrombin (34). It was proposed that the increased intracellular levels of syndecan-4 might act by perturbing the functional coordination of the biosynthetic enzymes. Loosening of the interaction between the 3-O-sulfotransferase and its polysaccharide substrate (Fig. 5) would be expected to interfere with 3-O-sulfation, as the enzyme was found to be strongly inhibited by other, more commonly occurring, saccharide sequences, such as the product of default modification (Fig. 1*B*) (33).

2-O-Sulfation of GlcA units has been demonstrated in a cell-free system and appears to occur concomitantly with the sulfation of IdoA, adjacent to at least one N-sulfated GlcN residue (Fig. 4; see ref 1). The mechanism behind the selection of certain GlcA units for 2-O-sulfation is unknown.

Proteins involved in the biosynthetic process

The proteins required to form a heparin or a HS PG include the appropriate core protein, enzymes that catalyze the formation and modification of GAG chains, and any auxiliary proteins that may be involved in the process.

Core proteins

The various core proteins known to carry heparin (serglycin) or HS (syndecans, perlecan, glypicans, and others) GAG chains have been discussed in previous reviews (1, 3, 4, 35, 36).

Enzymes

A deeper understanding of PG biosynthesis, and particularly its regulation, will depend on knowledge regarding the molecular characteristics of the enzymes involved and their genetics. Such information is accumulating due to purification and cloning of the enzymes, as well as through chemical mutagenesis of HS-producing cells (26). None of the enzymes involved in forming the linkage region tetrasaccharde sequence has yet been purified and cloned. A CHO cell mutant unable to produce HS was found to be defective with regard to both the GlcA-transferase and the GlcNAc-transferase reactions (37). Because the defect was presumably due to a single mutation, it was tentatively concluded that the two transferase reactions were catalyzed by a single enzyme protein. This assumption was supported by the identification of a ~70 kDa protein in bovine serum that promoted both reactions (38). The enzyme-deficient CHO cell line accumulated a protein-bound pentasaccharide composed of an α-GlcNAc unit bound to the linkage region tetrasaccharide (39), in accord with the notion that the "polymerase" differs from the GlcNAc-transferase that adds the first GlcNAc unit of the chain (28).

The notion of two distinct reactions being catalyzed by a single enzyme implies a rational means of promoting alternating events along a polymer chain. In fact, a similar arrangement applies to the two first polymer modification reactions, N-deacetylation and N-sulfation of GlcNAc units, which are both associated with the same ~110 kDa enzyme (refs 40–42 and references therein). The regulation of these reactions is essential, because the resultant distribution of N-acetyl and N-sulfate groups will control the followi~g modification reactions, and in fact will determine whether the final product will be classified a heparin or a HS. The N-deacetylase/N-sulfotransferase occurs in two distinct forms with partly different catalytic properties. One of these forms, first isolated from mouse mastocytoma (40), was associated with the biosynthesis of heparin, whereas the other, derived from rat liver (41), was implicated in HS generation. Both enzymes have been cloned (40–42), and comparison of the deduced amino-acid sequences showed that although the putative catalytic domains were closely related, other portions—in particular the N-terminal parts—were different. The two enzymes are encoded by transcripts of markedly different size (40, 42) that are related to separate genes (I. Eriksson, M. Kusche Gullberg, L. Kjellén, personal communication). Differential roles for these enzymes in regulating the N-deacetylation/N-sulfation process during heparin/HS biosynthesis were suggested by the finding that transfection of a HS-producing cell line with cDNA endocing the mast-cell N-deacetylase/N-sulfotransferase induced a drastic change of the N-substituent pattern of the HS produced by the cell toward that typical for heparin (43).

A ~52 kDa GlcA C5-epimerase has been purified to homogeneity from bovine liver (44). This enzyme cata-

lyzes the reversible conversion of GlcA to IdoA units, equilibrium favoring retention of the D-*gluco* configuration. However, whereas information on the kinetics of solubilized enzyme preparations may apply in part also to the intact biosynthetic system, the experimental conditions deviate drastically from those pertaining to GAG formation in the living cell. In this process, chain elongation and modification is completed within a few minutes or less, and individual reactions such as the GlcA C5-epimerization would not be allowed to approach equilibrium (45). In fact, studies of the formation of heparin in a mastocytoma microsomal fraction failed to show any "back-epimerization" (from IdoA to GlcA); the intact biosynthetic system is capable of delivering heparin chains in which ≥80% of the total hexuronic acid is IdoA. This remarkable efficiency is obviously, albeit still mysteriously, due to the mode of concerted interaction of the membrane-bound biosynthetic enzymes with their polymeric substrate.

Information regarding the molecular characteristics of the various O-sulfotransferases is still scanty. A ~60 kDa protein fraction, derived from detergent-solubilized mouse mastocytoma tissue, was found to catalyze both IdoA 2-O- and GlcN 6-O-sulfation, suggesting that these two reactions might also be associated with the same enzyme (46). On the other hand, cultured Chinese hamster ovary cells were found to release a GlcN 6-O-sulfotransferase into the medium while IdoA 2-O-sulfotransferase was retained by the cells (47). Purification of the former enzyme yielded two, 52 and 45 kDa, protein fractions. It cannot be concluded at present whether these discrepancies reflect actual differences between O-sulfotransferases that catalyze the corresponding reactions in heparin and HS biosynthesis or proteolytic processing of a common, membrane-bound O-sulfotransferase.

Auxiliary proteins

Scattered observations point indirectly to the involvement of additional auxiliary proteins in the biosynthetic process. The mouse mastocytomal N-deacetylase/N-sulfotransferase thus requires a polycationic cofactor for activity (40), whereas the corresponding rat liver enzyme does not (48). The endogenous polycation in the mast cell, apparently a polypeptide, may be replaced by synthetic polymers in assays of the purified enzyme. Moreover, analysis of cell mutants deficient in production of HS with high affinity for antithrombin implicated a regulatory component believed to somehow coordinate the action of the biosynthetic enzymes involved in generating the specific antithrombin-binding pentasaccharide sequence (49). Undoubtedly there are other, still undetected, proteins with similar regulatory functions. Such proteins need to be isolated, cloned, and characterized along with the biosynthetic enzymes in order to gain a better understanding of PG biosynthesis and its regulation. We may ultimately visualize the generation of artificial biosynthetic machineries, based on recombinant proteins assembled in appropriate membrane systems, that will enable the efficient formation of saccharide chains with specifically tailored structure. FJ

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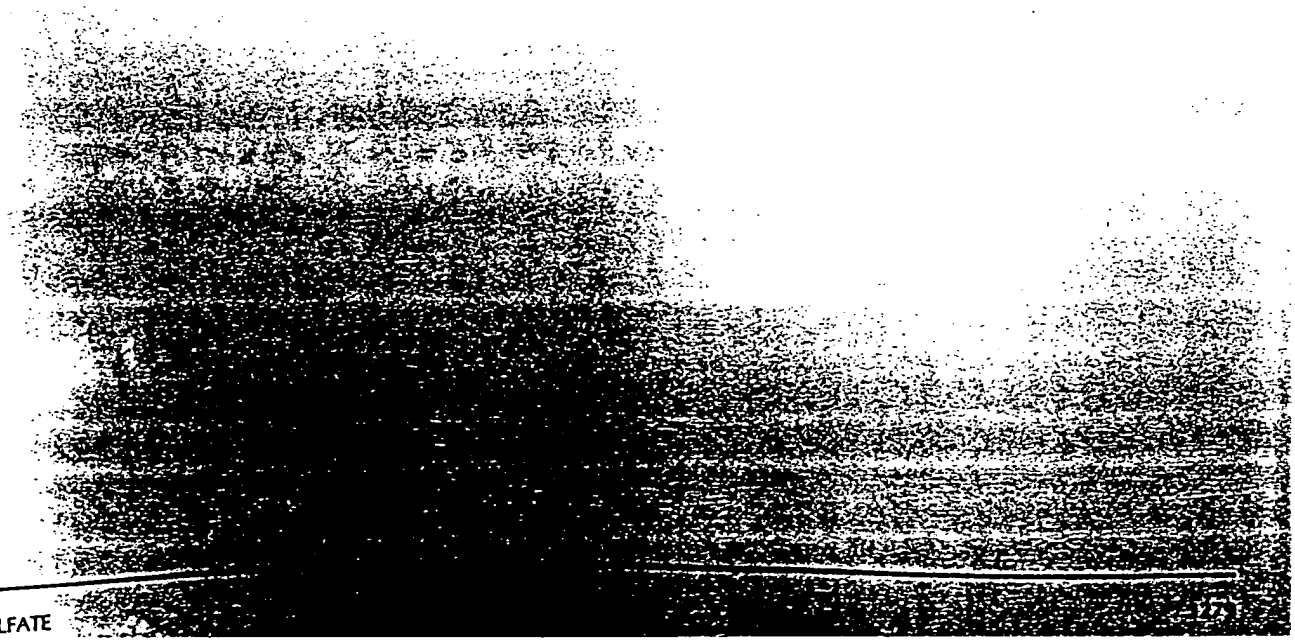

Proteoglycans in the nervous system

Arthur D. Lander

Massachusetts Institute of Technology, Cambridge, USA

Proteoglycans are ubiquitous cell-surface and secreted glycoproteins that are involved in diverse cellular behaviors. The identities of several nervous system proteoglycans, including many of the major species in the mammalian brain, have recently come to light. In addition, recent studies have given new insights into the roles of proteoglycans in nervous system development and function.

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Introduction

Proteoglycans (PGs) are found on the surfaces of all adherent cells, within intracellular vesicles, and in virtually all extracellular matrices (ECMs). They are evolutionarily ancient molecules, and play functional roles in the biology of growth factors, extracellular proteolysis, cell adhesion, lipoprotein metabolism, and virus entry into cells, as well as structural roles in maintaining the physical and mechanical properties of ECMs [1,2•,3,4].

Although many basic characteristics of PGs — their number, their structures, the exact nature of the functions they perform — are still slowly emerging, great progress has been made in recent years. With this recent burst of activity has come increasing recognition of the significance of PGs by neurobiologists, and increasing interest in the postulated roles PGs play in the nervous system. Some investigators have isolated monoclonal antibodies against nervous system molecules that have turned out to be PGs. Other investigators have become intrigued by the fact that many of the molecules that are thought to influence neuronal and glial cell behavior *in vivo*, especially during development, bind PGs. In the last few years, direct assaults on determining the structures of central nervous system (CNS) PGs have been undertaken by several groups. The purpose of this article is to review some of these recent results, and place them into the wider context of what PGs are, and how they are thought to function.

What are PGs?

A protein is called a PG if it contains a covalently attached glycosaminoglycan (GAG). GAGs are linear polysaccharides, typically 20–200 sugars in length, which are usually attached via a characteristic linkage region to serine residues. GAGs are built by the sequential addition of identical disaccharide units onto this linkage region. Only three types of disaccharide may be used, giving rise to three families of GAGs: the heparin/heparan family [D-glucuronic acid $\beta(1\rightarrow4)$ D-*N*-acetyl glucosamine $\alpha(1\rightarrow4)]_n$; the chondroitin/dermatan family [D-glucuronic acid $\beta(1\rightarrow3)$ D-*N*-acetyl galactosamine $\beta(1\rightarrow4)]_n$; and the keratan family [D-galactose $\beta(1\rightarrow4)$ D-*N*-acetyl glucosamine $\beta(1\rightarrow3)]_n$. The sugars of most GAGs are further chemically modified, typically in a sporadic fashion throughout the chain, by *O*-sulfation, *N*-deacetylation followed by *N*-sulfation, and/or epimerization (isomerization) of glucuronic acid to iduronic acid. Subsequently, GAGs are referred to as heparin, heparan sulfate (HS), chondroitin sulfate (CS), dermatan sulfate (DS) or keratan sulfate (KS). The heparin/HS distinction and the CS/DS distinction only reflect differences in level of modification (i.e. heparin is more highly modified than most HS species; DS contains much more iduronate than CS). As each disaccharide in a GAG chain may be modified to a different degree, the large scale structures of GAGs can be exceedingly complex (e.g. in HS, which can be modified in up to five ways, a hexasaccharide can theoretically have over 30,000 possible chemical structures).

Products of several gene families, including secreted and membrane-inserted polypeptides, act as the core proteins of major PGs (Table 1). Some bear as few as one GAG chain, whereas others have over a hundred. Although the signals that specify whether a serine residue will bear a GAG are partially understood [2•], it is not known what controls the type of GAG synthesized: examples exist of cores that always bear one type of GAG, cores that bear different GAGs at different sites, and cores that bear different GAGs depending on the cell type in which they are expressed.

Abbreviations

CNS—central nervous system; **CS**—chondroitin sulfate; **DS**—dermatan sulfate; **ECM**—extracellular matrix; **FGF**—fibroblast growth factor; **GAG**—glycosaminoglycan; **HS**—heparan sulfate; **KS**—keratan sulfate; **NCAM**—neural cell adhesion molecule; **NgCAM** - neuron-glial cell adhesion molecule; **PG** —proteoglycan.

Table 1. Cloned PG core proteins.[a]

Cell-surface PGs	
	Syndecan family
	Syndecan (Syndecan-1)
	Fibroglycan (Syndecan-2)
	N-Syndecan Syndecan-3
	Ryudican Amphiglycan Syndecan-4
	Glypican family
	Glypican
	Cerebroglycan
	NG-2
	'Part-time PGs'[b]
ECM PGs	
	Aggrecan family
	Aggrecan
	Versican
	Neurocan
	Small, interstitial PG family
	Decorin
	Biglycan
	Fibromodulin
	Lumican
	Perlecan
	Type IX Collagen
Intravesicular PGs	
	Serglycin
	SV2

[a]Only shown are the obligate PG core proteins, i.e. those that invariably bear GAG chains. A small number of other cell-surface proteins bear GAG chains in some cells, but not others. [b]These 'part-time' PGs include CD44 and the type III transforming growth factor (TGF)-β receptor (reviewed in [1,2•,53]).

Cell surface PGs of the CNS

Early progress toward identifying cell surface PGs of the brain was made by Margolis' group, who detected a single major HSPG in adult brain membranes [5]. Later, Herndon and I [6] found evidence for CSPGs and other, less abundant, HSPGs in adult brain membranes, as well as additional major HSPGs that are present only during development. In the past year, the core proteins of several of these have been identified.

Glypican

Glypican was first identified as a surface HSPG core protein of human fibroblasts [7]. The mature polypeptide is ~53 kDa and is anchored in the plasma membrane by covalently attached glycosylphosphatidylinositol. Both the adult brain HSPG identified by Klinger *et al.* [5], and brain HSPG M12 identified by us [6], are the rat form of glypican ([8]; ED Litwack, CS Stipp, A Kumbasar, AD Lander, unpublished data). *In situ* hybridization studies in the adult brain and spinal cord indicate that glypican mRNA is expressed primarily, if not exclusively, by projection neurons in many, but not all parts of the CNS (ED Litwack, CS Stipp, A Kumbasar, AD Lander, unpublished data) (see Table 2). In the embryo, glypican is also strongly expressed in ventricular zones (regions undergoing neural precursor proliferation) throughout the neuraxis (Fig. 1).

Cerebroglycan

Cerebroglycan, previously called PG M13 [6], is an HSPG with a ~58 kDa core protein, and was first detected in the embryonic and newborn — but not adult — rat brain. Like glypican, it is glycosylphosphatidylinositol-anchored. In fact, glypican and cerebroglycan define a family of lipid-anchored HSPG cores, based on amino acid sequence similarity (CS Stipp, ED Litwack, AD Lander, unpublished data) (see Table 2). *In situ* hybridization studies indicate that cerebroglycan is transiently expressed by postmitotic neurons throughout the CNS (Fig. 1). Evidently, cerebroglycan mRNA appears in neurons shortly after terminal mitosis and disappears after neuronal migration and axon growth have been completed. Interestingly, cerebroglycan is not expressed outside the nervous system.

N-syndecan

N-syndecan (or syndecan-3) is one of four members of the syndecan family of transmembrane core proteins (Table 1). These polypeptides have short (~34 amino acids) cytoplasmic domains that are highly conserved among all family members, and overall sizes varying from 20 kDa (syndecans-2 and -4) to ≥ 42 kDa (syndecan-3). Their extracellular domains are poorly conserved among the different family members, or even for the same syndecan in different mammalian species. *N*-syndecan was cloned by Carey *et al.* [9••], who identified it in rat Schwann cell membranes (see Table 2). High levels of *N*-syndecan mRNA are also found in neonatal rat brain, as well as in many sites outside the nervous system. Expression of this molecule in rat brain peaks at birth, declining to undetectable levels thereafter. Early immunohistochemical studies suggest that this PG is associated with fiber tracts, but it is not yet known whether its source is neuronal or glial.

Syndecan-2

Syndecan-2, also known as fibroglycan, another member of the syndecan family, has not yet been isolated from the brain, but its mRNA has been found there (see Table 2). Based on electrophoretic behavior, syndecan-2 may correspond to brain PG M14 [6].

Table 2. PGs of the mammalian CNS.[a]

Name	Family	GAG	CNS Expression
Syndecan-3	Syndecan	HS	Transiently expressed in perinatal brain; widespread [9••]
Glypican[b]	Glypican	HS	Neuroepithelium; certain adult projection neurons [8][c]
Cerebroglycan[b]	Glypican	HS	Transiently expressed by newly post-mitotic neurons[d]
NG-2	NG-2	CS	O-2A progenitors [10]
Syndecan-2	Syndecan	HS	Unknown [2•]
Neurocan (1D1)	Aggrecan	CS	White matter of developing cerebellum; molecular layer of adult cerebellum. Mostly intracellular in adult [12••,18]
Versican	Aggrecan	CS	White matter [13•]
Aggrecan	Aggrecan	CS	Embryonic chick brain [14•]
Cat-301	Aggrecan?	CS	Subsets of neurons, cerebellum and spinal cord [15•,20]
PG-T1	?	CS	Widespread [16••,17•]
3H1	?	CS KS	Similar to neurocan [18]
3F8	?	CS	Concentrated in molecular layer of developing and adult cerebellum [18]
6B4	?	CS	Cerebellar and brainstem projection neurons [19•]
Unnamed	?	HS	Transient, in CNS fiber tracts [26••]
SV2 antigen	SV2	KS	Synaptic vesicles [27••]

[a]PGs are referred to by the names of their core proteins, and are grouped according to whether they are cell surface, extracellular matrix/soluble, or intravesicular molecules (see text). In many cases, information on CNS distribution has been based on the examination of only a few brain regions, and is therefore incomplete. [b]Data on distribution of glypican and cerebroglycan are based on *in situ* hybridization; most other data were obtained using antibodies. [c]ED Litwack, CS Stipp, A Kumbasar, AD Lander, unpublished data. [d]CS Stipp, ED Litwack, AD Lander, unpublished data.

NG2

NG2 is a transmembrane CSPG with a 300 kDa core protein [10]. In the brain it is associated with a population of glial precursor cells, the O-2A progenitors (see Table 2), that give rise to oligodendrocytes and a type of astrocyte. The very large core protein of NG2 suggests that it may serve functions other than just bearing CS chains. One such function appears to be the binding of type VI collagen [11].

ECM and 'soluble' PGs of the CNS

Many PGs can be extracted from the brain using physiological buffers without detergent; others require high salt or denaturing conditions. Although it has been argued that some of these molecules may reside in the cytoplasm of cells, most are probably loosely associated with the ECM.

Most of the PGs in these categories contain CS as their major GAG. Neurocan, a recently cloned CSPG, has a 136 kDa core protein, and contains ~3 CS chains [12••]. Its protein sequence places it in a family with aggrecan — the major ECM PG of cartilage — and versican, an ECM PG first found associated with fibroblasts. Like these other PGs, neurocan binds the ECM polysaccharide hyaluronic acid via a protein domain that is highly conserved in all three family members. Recent evidence suggests that versican and aggrecan are themselves expressed in the human and chicken brain, respectively [13•,14•]. The Cat-301 antigen is yet another large brain CSPG that binds hyaluronic acid, and immunological evidence suggests that it is related to aggrecan [15•]. One additional hyaluronic acid-binding CSPG, the T1 antigen, has been identified in brain, but at least the hyaluronic acid-binding region of this molecule is apparently unrelated to those of the aggrecan family [16••,17•]. Still other brain CSPGs have been identified with monoclonal antibodies, and remain to be fully characterized [14•,18,19•].

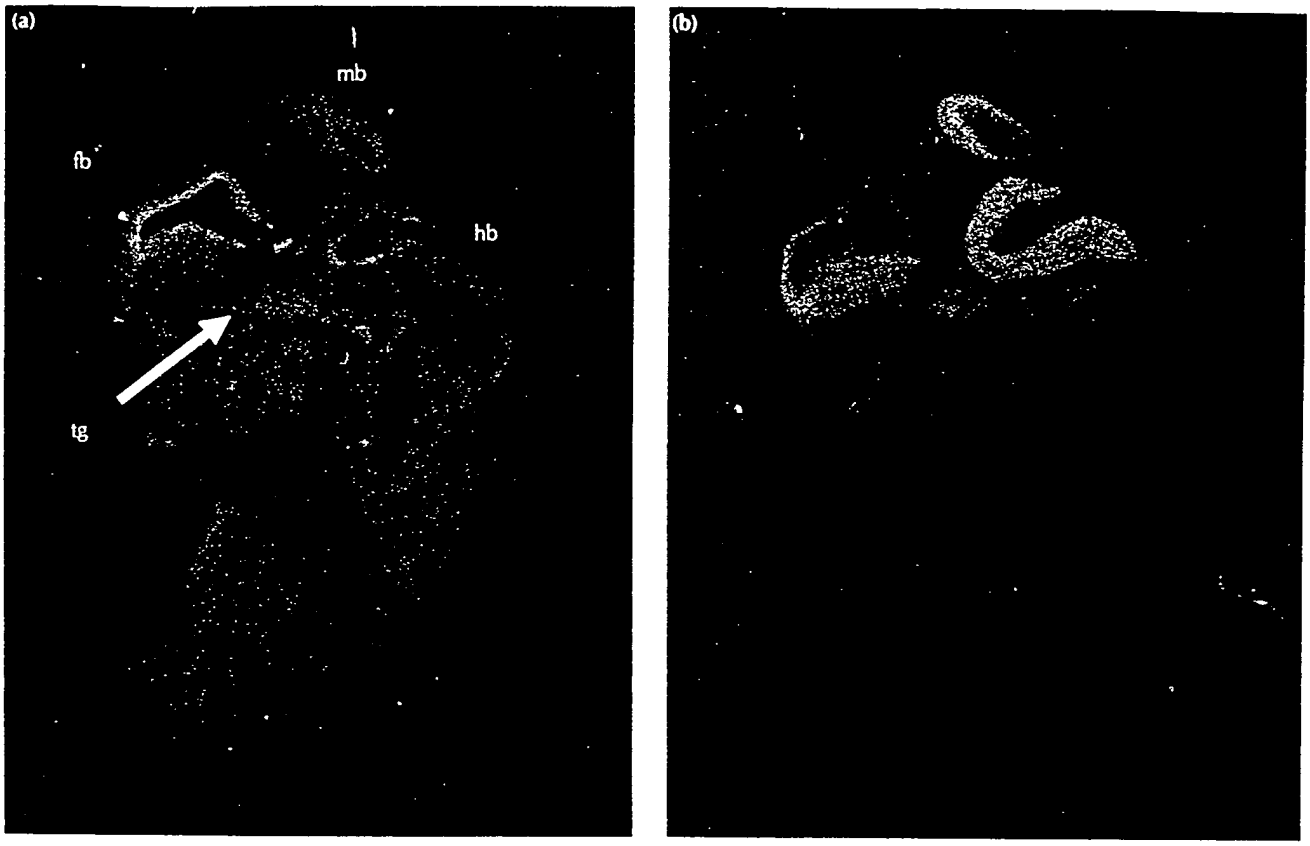

Fig. 1. Expression of glypican and cerebroglycan in the rat embryo. Adjacent sections of embryonic day 14 rats were hybridized with radiolabeled RNA probes specific for **(a)** glypican and **(b)** cerebroglycan mRNA. The images are reverse contrast prints of the resulting autoradiograms. Glypican expression is found throughout the embryo, but is particularly strong in the ventricular zones of the developing CNS. In contrast, cerebroglycan mRNA, which is found only in neural tissue, is not detected in ventricular zones but is found in the layers of immature neurons that form around those zones. In the adult brain, glypican is expressed by subpopulations of neurons, whereas cerebroglycan is absent. fb—forebrain; mb—midbrain; hb—hindbrain; tg—trigeminal ganglion.

The distributions of these CSPGs vary from remarkably uniform throughout the brain (PG T1) to remarkably cell type- and de opmental stage-specific. For example, Cat-301 appears around certain subsets of neurons only after activity-dependent critical periods in their development [20]. Another is transiently expressed during axon outgrowth by several types of neurons involved in the cerebellar mossy fiber system [19•].

As information on the distribution of these CSPG and CS KSPG core proteins accumulates, so has information on the distribution of different types of CS and KS chains. Several investigators have observed remarkable cell-type specificity in the binding of anti-CS and anti-KS monoclonal antibodies to brain sections (e.g. [21,22]). During cerebral cortex development, CS is found in the early proliferative neuroepithelium, then later in the marginal zone and subplate regions [23]. With the exception of the subplate, many of the locations of CS expression during development correlate with sites where axons do not grow. For example, CS, as well as KS, are strongly expressed in the roof plate of the spinal cord [24,25].

Recently, a report of an HSPG in brain ECM appeared [26••]. This molecule is found in basal laminae outside of and surrounding the chicken brain, but is also expressed transiently in many developing CNS axon tracts. The core protein size (250 kDa) and basal laminar distribution of this PG are reminiscent of perlecan, a major basement membrane PG, but perlecan itself is not found in S axon tracts.

Synaptic vesicle PGs

It has long been known that a PG is a major component of synaptic vesicles isolated from the electric organs of fishes. This PG was recently shown to be a transmembrane KSPG, and appears to be involved in acetylcholine transport into vesicles [27••]. Immunochemical data suggest that this molecule is present in many other types of synaptic vesicles, and might therefore play an important general role in transmitter uptake.

Roles of PGs in the nervous system

Insights into the functions of PGs in the nervous system have come by many routes, direct and indirect, and many of the conclusions are still somewhat preliminary. Highlights of what has been learned are summarized below.

The functions of a family of growth factors are dependent on PGs

All members of the fibroblast growth factor (FGF) family bind GAGs of the heparin/HS class, and apparently must do so to be biologically active [28,29]. Recent studies support a model in which cell-surface HSPGs bind both FGFs and FGF receptors simultaneously, facilitating their interaction [30]. It is known that at least three FGFs — FGF-1, -2 and -5 — are expressed in the nervous system and exert trophic effects on several classes of neurons [31–34,35••]. Recently, Nurcombe *et al.* [35••] have suggested that differences in the type of HS carried by a single core protein can render early neuroepithelial cells selectively responsive either to FGF-1 or to FGF-2. This proposition is supported by evidence in other systems that HS structure can impart specificity to HSPG function (e.g. [36,37•,38,39•]).

The kinetics of action of a family of protease inhibitors are dependent on PGs

The structurally related molecules antithrombin III, heparin cofactor II, and protease nexin I all bind and inactivate certain serine proteases (e.g. thrombin) much more rapidly when appropriate GAGs are present. To a large extent, GAGs act by simultaneously binding both protease and protease inhibitor, confining them to the same locality and thereby facilitating their interaction [38]. Of interest to neurobiologists, protease nexin I is abundantly expressed in the CNS, and is thought to regulate neurite outgrowth and neuronal migration [40].

Cell surface PGs participate in establishing cell–cell and cell–ECM contacts

Although cell surface PGs can apparently be the sole receptors for attachment to certain substrata [37•], PGs usually facilitate interactions mediated through other receptors, such as integrin-dependent cell attachment to ECM molecules [41], and neural cell adhesion molecule (NCAM)-dependent cell–cell adhesion [42,43]. A recent study suggests that cell surface HSPGs are especially important for the interaction of neural cells with fibronectin [44•]. As ECM and cell adhesion molecules are thought to provide important navigational cues to growing axons, the involvement of PGs with such molecules suggests a potential role for PGs in axon guidance. Recent studies in insects support this idea [45••].

ECM PGs regulate cell–cell and cell–matrix interactions

The core protein of at least one PG, perlecan, supports integrin-mediated cell attachment [46]. In contrast, several PGs inhibit the biological activities of ECM and cell adhesion molecules, at least *in vitro*. For example, adsorbed CSPGs or CS KSPGs can render culture substrata inhospitable for neurite growth [24,25]. Soluble CSPGs from rat brain also inhibit neurite outgrowth by PC12 cells [47]. Neurocan and the 3F8 CSPG of rat brain (but not aggrecan) inhibit homophilic NCAM and neuron-glial cell adhesion molecule (NgCAM)-binding [48•]. A HSPG released by Schwannoma cells specifically blocks the neurite outgrowth-promoting activity of laminin [49]. In some of these cases, the GAG chains of the PGs are required for these actions [24,25,49]; in others they are not [47,48•]. It is not yet known whether these phenomena are direct actions of PGs on neurons, or reflect effects of PGs on the physical characteristics of the culture substratum, so caution must be used in extrapolating these results to *in vivo* settings. Nonetheless, the distributions of some CSPGs are consistent with a 'barrier' function *in vivo* (see above). For example, in the developing retina a receding wave of CS expression marks a front of centripetally directed axons, suggesting that axons are guided by their avoidance of CS. Intriguingly, a CS-degrading enzyme disrupts the timing and direction of retinofugal axons in the developing rat retina [50••].

PGs are involved in the assembly of ECM, and act as binding sites for molecules that associate with the ECM

PGs bind virtually every major ECM component. In addition, molecules such as growth factors (e.g. FGFs) and enzymes (e.g. synaptic acetylcholinesterase) are often immobilized in ECMs through interactions with HSPGs [1,51]. The importance of PGs in ECM structure and function is illustrated by a muscle cell line that is defective in GAG biosynthesis [52•]. This cell line produces an abnormal basal lamina and, probably as a consequence, fails to form acetylcholine receptor clusters. The cells also fail to form such clusters in response to agrin, a GAG-binding ECM molecule that potently induces receptor clusters on normal muscle cells, and is thought to be involved in synaptogenesis *in vivo*.

Conclusions

Although much still needs to be learned about nervous system PGs, the identities of many of the major species in the brain are now known. Tracking down the functions of these molecules will probably not be easy. Their biological activities are likely to reside in their capacity to regulate, possibly in subtle ways, the functions of the molecules they bind. Moreover, the repertoire of molecules they bind will probably depend in part on the precise structures of their GAG chains, structures which defy easy analysis. Nevertheless, PGs are likely to continue to receive increasing attention in neurobiology, as their *in vivo* distributions and *in vitro* activities suggest that they are widely involved in nervous system development and function.

Acknowledgements

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The cloning of neurocan is described. The cDNA that was obtained encodes a protein similar to aggrecan and versican, including an amino-terminal hyaluronate binding domain, and carboxy-terminal epidermal growth factor (EGF)-like, lectin-like and complement regulatory-like domains. Evidence is presented that the adult brain contains only a truncated form of neurocan, lacking the amino-terminal region.

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One of only a few studies to look at PGs in the avian brain. Aggrecan and its mRNA are found to be transiently expressed in the embryonic chick brain. Unlike cartilage aggrecan, the brain form contains only CS and not KS. Also detected in chick brain was a PG with a similar core size, but that can be distinguished from aggrecan by the presence of both CS and KS chains, reactivity with the HNK-1 monoclonal antibody, and lack of developmental regulation.

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The Cat-301 immunoreactive CSPG of brain is shown to have properties similar to those of aggrecan. Also, authentic aggrecan from cartilage is shown to react with Cat-301, as well as to another monoclonal antibody that recognizes the same brain PG as Cat-301. Nevertheless, the Cat-301 antigen can be distinguished from aggrecan by virtue of containing few, if any, KS chains and having a lower buoyant density (indicative of a lower carbohydrate protein ratio) than aggrecan. The data suggest that Cat-301 is either a modified form of aggrecan, or a new member of the aggrecan family.

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AD Lander, Department of Brain and Cognitive Sciences, E25-435, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA.

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